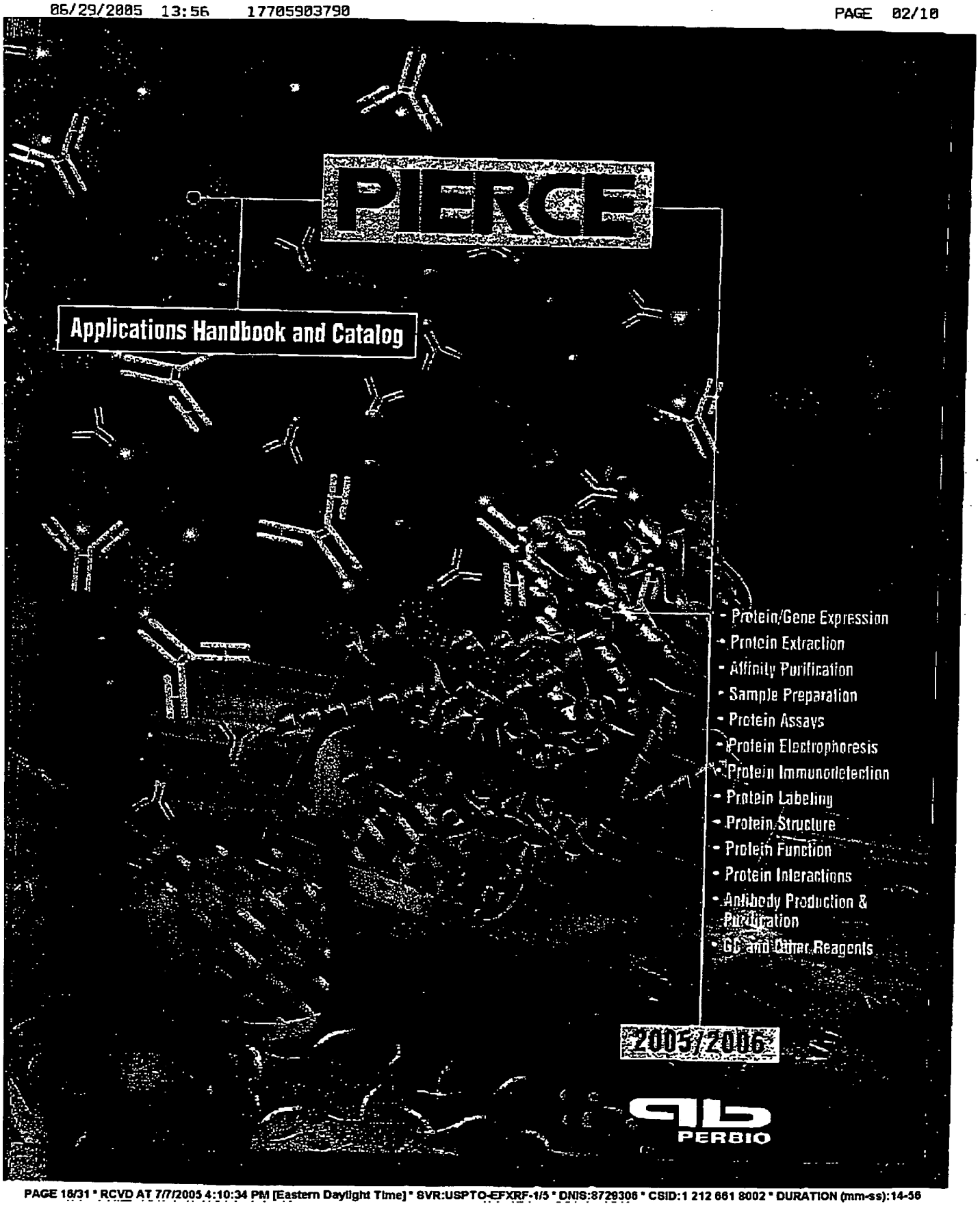
EXHIBIT 1

Best Available Copy


PIERCE
Applications Handbook and Catalog
- Protein/Gene Expression
- Protein Extraction
- Affinity Purification
- Sample Preparation
- Protein Assays
- Protein Electrophoresis
- Protein Immunodetection
- Protein Labeling
- Protein Structure
- Protein Function
- Protein Interactions
- Antibody Production & Purification
- GC and Other Reagents
2005/2006
PERBIO

Protein Structure – Table of Contents

Technical Information

9

Protein Structure – Table of Contents

Technical Information *continued*

Product Information

Protein Structure – Cross-linking

Introduction to Cross-linking

What is Cross-linking?

Cross-linking is the process of chemically joining two or more molecules by a covalent bond. Cross-linking reagents contain reactive ends to specific functional groups (primary amines, sulfhydryls, etc.) on proteins or other molecules. Cross-linking reagents have been used to assist in determination of near-neighbor relationships, three-dimensional structures of proteins, and molecular associations in cell membranes. They also are useful for solid-phase immobilization, hapten-carrier protein conjugation, preparing antibody-enzyme conjugates, immunotoxins and other labeled protein reagents. Other uses include modification of nucleic acids, drugs and solid surfaces.

Conformational changes of proteins associated with a particular interaction may be analyzed by performing cross-linking studies before and after the interaction occurs. Comparing cross-linkers with different arm lengths for success of conjugation can provide information about the distances between interacting molecules. By examining which cross-linkers effectively conjugate to particular domains of a protein, information may be obtained about conformational changes that hindered or exposed amino acids in the tertiary and quaternary structure.

The use of cross-linkers has made the study of surface receptors much easier. By derivatizing a receptor with a cross-linker before or after contact with the ligand, it is possible to isolate the receptor-ligand complex. The use of radioiodinatable cross-linkers makes it possible to identify a particular receptor by autoradiographic detection.

Homo- and Heterobifunctional Cross-linkers

Cross-linkers can be either homobifunctional or heterobifunctional. Homobifunctional cross-linkers have two identical reactive groups and often are used in one-step reaction procedures to cross-link proteins to each other or to stabilize quaternary structure. Even when conjugation of two different proteins is the goal, one-step cross-linking with homobifunctional reagents often results in self-conjugation, intramolecular cross-linking and/or polymerization.

Heterobifunctional cross-linkers possess two different reactive groups that allow for sequential (two-stage) conjugations, helping to minimize undesirable polymerization or self-conjugation. Heterobifunctional reagents can be used when modification of amines is problematic. Amines are sometimes present at the active sites of proteins and modification of these may lead to activity loss. Other moieties such as sulfhydryls, carboxyls, phenols and carbohydrates may be more appropriate targets. A two-step strategy allows a protein that can tolerate the modification of its amines to be coupled to a protein or other molecule having different accessible groups. Cross-linkers that are amine-reactive at one end and sulfhydryl-reactive at the other end are especially useful in this regard.

In sequential procedures, heterobifunctional reagents are reacted with one protein using the most labile group of the cross-linker first. After removing excess nonreacted cross-linker, the modified first protein is added to a solution containing the second protein where reaction through the second reactive group of the cross-linker occurs. The most widely-used heterobifunctional cross-linkers are those having an amine-reactive succinimidyl ester (i.e., NHS-ester) at one end and a sulfhydryl-reactive group on the other end. The sulfhydryl-reactive groups are usually maleimides, pyridyl disulfides and α-haloacetyls. The NHS-ester reactivity is less stable in aqueous solution and is usually reacted first in sequential cross-linking procedures. NHS-esters react with amines to form amide bonds. Carbodiimides are zero-length cross-linkers (e.g., EDC, Product # 22980, 22981) and effect direct coupling between carboxylates (–COOH) and primary amines (–NH_2) and have been used in peptide synthesis, hapten-carrier protein conjugation, subunit studies and protein-protein conjugation.

Other heterobifunctional reagents have one reactive group that is photoreactive rather than thermoreactive. These have distinct advantages in protein:protein interaction studies and in cases where the availability of thermoreactive targetable functional groups is unknown. This reactivity allows for specific attachment of the labile thermoreactive group first; subsequently, conjugation to any adjacent N-H or C-H sites may be initiated through the photoreactive group by activation with UV light.

The reactivity of the photochemical reagent allows for formation of a conjugate that may not be possible with a group-specific reagent. The efficiency of photoreactive cross-linkers is low, and yields of 10% are considered acceptable. However, SFAD (Product # 27719) is a photoactivatable reagent that contains a perfluorophenyl azide with an insertion efficiency of 70%.

How to Choose a Cross-linker

Cross-linkers are selected on the basis of their chemical reactivities (i.e., specificity for particular functional groups) and compatibility of the reaction with the application. The best cross-linker to use for a specific application must be determined empirically. Cross-linkers are chosen based on the following characteristics:

- Chemical specificity
- Spacer arm length
- Reagent water-solubility and cell membrane permeability
- Same (homobifunctional) or different (heterobifunctional) reactive groups
- Thermoreactive or photoreactive groups
- Reagent cross-links cleavable or not
- Reagent contains moieties that can be radiolabeled or tagged with another label

Cross-linkers contain at least two reactive groups. Functional groups that can be targeted for cross-linking include primary amines, sulfhydryls, carbonyls, carbohydrates and carboxylic acids (Table 1). Coupling also can be nonselective using a photoreactive phenyl azide cross-linker. Our web site contains a cross-linker selection guide by which the above-listed parameters may be chosen and a list of available cross-linkers with those features generated. A cross-linker selection guide appears at the beginning of the cross-linker product listing in the catalog section.

Table 1. Reactive Cross-linker Groups and Their Functional Group Targets

Reactive Group	Functional Group
Aryl Azide	Non-selective (or primary amine)
Carbodiimide	Amine/Carboxyl
Hydrazide	Carbohydrate (oxidized)
Hydroxymethyl Phosphine	Amine
Imidoester	Amino
Isocyanate	Hydroxyl (non-aqueous)
Maleimide	Sulfhydryl
NHS-ester	Amine
PFP-ester	Amine
Psoralen	Thymine (photoreactive intercalator)
Pyridyl Disulfide	Sulfhydryl
Vinyl Sulfone	Sulfhydryl, amino, hydroxyl

Protein Structure – Cross-linking

Often different spacer arm lengths are required because steric effects dictate the distance between potential reaction sites for cross-linking. For protein:protein interaction studies, the proximity between reactive groups is difficult to predict. Usually, a cross-linker with a short spacer arm (4-8 Å) is used first and the degree of cross-linking determined. A cross-linker with a longer spacer arm may then be used to optimize cross-linking efficiency. Short spacer arms are often used in intramolecular cross-linking studies, and intermolecular cross-linking is favored with a cross-linker containing a long spacer arm. Often cross-linkers that are cleavable, non-cleavable and have various spacer arm lengths are used to obtain a complete analysis of protein structure.

General Reaction Conditions

In many applications, it is necessary to maintain the native structure of the protein complex, so cross-linking is most often performed using mild pH and buffer conditions. Furthermore, optimal cross-linker-to-protein molar ratios for reactions must be determined. Depending on the application, the degree of conjugation is an important factor. For example, when preparing immunogen conjugates, a high degree of conjugation is desired to increase the immunogenicity of the antigen. However, when conjugating to an antibody or an enzyme, a low-to-moderate degree of conjugation may be optimal so that biological activity of the protein is retained. The number of functional groups on the protein's surface is also important to consider. If there are numerous target groups, a lower cross-linker-to-protein ratio can be used. For a limited number of potential targets, a higher cross-linker-to-protein ratio may be required. Furthermore, the number of components should be kept low or to a minimum because conjugates consisting of more than two components are difficult to analyze and provide less information on spatial arrangements of protein subunits.

Cross-linker Reactivities

Imidoesters

Imidoester cross-linkers react with primary amines to form amidine bonds. The resulting amidine is protonated and therefore, has a positive charge at physiological pH (Figure 2). Imidoester homobifunctional cross-linkers have been used to study protein structure, molecular associations in membranes, and to immobilize proteins onto solid-phase supports. They also have been examined as a substitute for glutaraldehyde for tissue fixation. Imidoesters can penetrate cell membranes and cross-link proteins within the membrane to study membrane composition, structure and protein:protein and protein:lipid interactions. These cross-linkers have also been used to determine or confirm the number and location of subunits within multi-subunit proteins. In these experiments, large molar excesses of cross-linker (100-1,000 fold) and low concentrations of protein (≤1 mg/ml) are used to favor intramolecular over intermolecular cross-linking.

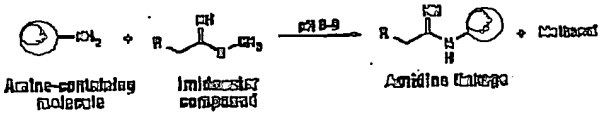


Figure 2. Imidoester reaction scheme.

Imidoester cross-linkers react rapidly with amines at alkaline pH but have short half-lives. As the pH becomes more alkaline, the half life and reactivity with amines increases; therefore, cross-linking is more efficient when performed at pH 10 than at pH 8. Reaction conditions below pH 10 may result in side reactions, although amidine formation is favored between pH 8-10. Studies using monofunctional alkyl imidates reveal that at pH <10 conjugation can form with just one imidoester functional group. An intermediate N-alkyl imidate forms at the lower pH range and will either cross-link to another amine in the immediate vicinity, resulting in N,N'-amidine derivatives, or it will convert to an amidine bond. At higher pH, the amidine is formed directly without formation of an intermediate or side product. Extraneous cross-linking that occurs below pH 10 sometimes interferes with interpretation of results when thiol-cleavable dilmidoesters are used.

Although these cross-linkers are still used in protein subunit studies and solid-phase immobilization, the amidine bonds formed by imidoester cross-linkers are reversible at high pH, and therefore, the more stable and efficient NHS-ester cross-linkers have steadily replaced them.

N-Hydroxysuccinimide Esters (NHS Esters)

NHS esters yield stable products upon reaction with primary amines with relatively efficient coupling at physiological pH. Accessible α-amine groups present on the N-termini of proteins and ε-amines on lysine residues react with NHS esters and form amide bonds. A covalent amide bond is formed when the NHS-ester cross-linking agent reacts with a primary amine, releasing *N*-hydroxysuccinimide (Figure 3).

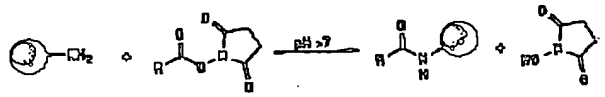


Figure 3. NHS-ester reaction scheme.

Hydrolysis of the NHS-ester competes with the primary amine reaction. Hydrolysis rate increases with increasing pH and occurs more readily in dilute protein solutions. Studies performed on NHS-ester compounds indicate the half-life of hydrolysis for a homobifunctional NHS-ester is 4-5 hours at pH 7.0 and 0°C in aqueous environments free of primary amines. This half-life decreases to 10 minutes at pH 8.6 and 4°C. The extent of the NHS-ester hydrolysis in aqueous solutions free of primary amines may be measured at 260 nm. An increase in absorbance at this wavelength is caused by the release of NHS. The molar extinction coefficient of NHS released by hydrolysis and reaction with a nucleophile is 8.2×10^3 M^{-1} cm^{-1} at 260 nm at pH 9.0. The molar extinction coefficient for the NHS ester in 50 mM potassium phosphate buffer, pH 6.5 is 7.5×10^3 M^{-1} cm^{-1} at 260 nm.

NHS-ester cross-linking reactions are most commonly performed in phosphate, bicarbonate/carbonate, HEPES or borate buffers at concentrations between 50-200 mM. Other buffers may also be used if they do not contain primary amines. HEPES, for example, can be used because it contains only tertiary amines. Primary amines are present in the structure of Tris, which makes it an unacceptable buffer for NHS-ester reactions. A large excess of Tris at neutral-to-basic pH may be added at the end of a NHS ester reaction to quench it. Glycine also contains a primary amine and may be used in a similar manner. The NHS-ester reactions are typically performed between pH 7 and 9 and at 4°C to room temperature from 30 minutes to 2 hours. Reaction times at 4°C are increased 4-fold from room temperature incubation times to produce similar efficiencies. NHS esters are usually used at 2- to 50-fold molar excess to protein depending on the concentration of the protein. Typically, the concentration of the cross-linker can vary from 0.1-10 mM. The protein concentration should be kept above 10 µM (50-100 µM is optimal) because more dilute protein solutions result in excessive hydrolysis of the cross-linker.

Protein Structure – Cross-linking

NHS esters can be grouped into two separate classes with essentially identical reactivity toward primary amines: water-soluble and water-insoluble. Water-soluble NHS esters have a sulfonate ($-SO_3$) group on the *N*-hydroxysuccinimide ring. They are advantageous when the presence of organic solvents cannot be tolerated. The reaction with the sulfo-NHS esters is usually performed in 100% aqueous solutions; however, it is possible to achieve greater solubility when the reagent is dissolved in organic solvents such as DMSO (Product # 20686). The water-soluble NHS-ester cross-linkers are used for cell surface conjugation because they will not permeate the membrane. Sulfonated NHS-ester cross-linkers are supplied as sodium salts and are soluble in water to a concentration of at least 10 mM.

The solubility of the NHS esters will vary with buffer composition. The non-sulfonated forms of NHS-ester reagents are water-insoluble and are first dissolved in water-miscible organic solvent, such as DMSO (Product # 20684, 20686) and DMF (Product # 20672, 20673), then added to the aqueous reaction mixture. The water-insoluble cross-linkers do not possess a charged group and are lipophilic and membrane-permeable. Cross-linking reactions with the water-insoluble NHS esters are typically performed with a solvent carryover of 0.5-10% final volume in the aqueous reaction. In some cases, cross-linking proteins with NHS esters may result in loss of biological activity that may be a result of conformational change of the protein when the NHS-ester cross-linker reacts with primary amines on the molecule's surface. Loss of activity may also occur when any of the lysine groups involved in binding a substrate (in the case of an enzyme) or an antigen (in the case of an antibody) are modified by the cross-linker.

Maleimides

Coupling through sulfhydryl groups is advantageous because it can be site-directed, yield cleavable products and allow for sequential coupling. A protein in a complex mixture can be specifically labeled if it is the only one with a free sulfhydryl group on its surface. If there are insufficient quantities of free sulfhydryls, they can be generated by reduction of disulfide bonds. Alternatively, sulfhydryls can be introduced into molecules through reaction with primary amines using 2-Iminothiolane or Traut's Reagent (Product # 26101), SATA (Product # 26102) or SPDP (Product # 21857).

The maleimide group reacts specifically with sulfhydryl groups when the pH of the reaction mixture is between pH 6.5 and 7.5 and forms a stable thioether linkage that is not reversible (Figure 4). At neutral pH, maleimides react with sulfhydryls 1,000-fold faster than with amines, but at pH >8.5, the reaction favors primary amines. Maleimides do not react with tyrosines, histidines or methionines. Hydrolysis of maleimides to a non-reactive maleamic acid can compete with thiol modification, especially above pH 8.0. Thiols must be excluded from reactions buffers used with maleimides because they will compete for coupling sites. Excess maleimides can be quenched at the end of a reaction by adding free thiols. EDTA can be included in the coupling buffer to minimize oxidation of sulfhydryls.

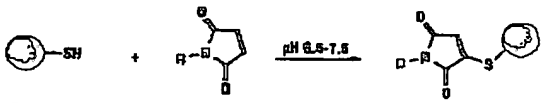


Figure 4. Maleimide reaction scheme.

Haloacetyls

The most commonly used α-haloacetyl cross-linkers contain the iodoacetyl group that react with sulfhydryl groups at physiological pH. The reaction of the iodoacetyl group with a sulfhydryl proceeds by nucleophilic substitution of iodine with a thiol producing a stable thioether linkage (Figure 5). Using a slight excess of the iodoacetyl group over the number of sulfhydryl groups at pH 8.3 ensures sulfhydryl selectivity. In the absence of free sulfhydryls, or if a large excess of iodoacetyl group is used, the iodoacetyl group can react with other amino acids. Imidazoles can react with iodoacetyl groups at pH 6.9-7.0, but the incubation must proceed for longer than one week.

Histidyl side chains and amino groups react in the unprotonated form with iodoacetyl groups above pH 5 and pH 7, respectively. To limit free iodine generation, which has the potential to react with tyrosine, histidine and tryptophan residues, perform iodoacetyl reactions and preparations in the dark. Avoid exposure of iodoacetyl compounds to reducing agents. Available NHS-ester haloacetyl cross-linkers are listed in Table 2.

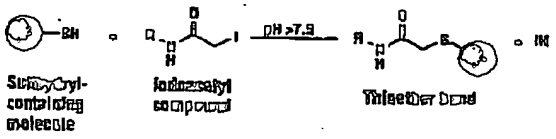


Figure 5. Active halogen reaction scheme.

Table 2. Pierce's NHS-ester Haloacetyl Cross-linkers

Reagent	Reactivity	Product #
SIA	Amine/Sulfhydryl	22349
SIAB	Amine/Sulfhydryl	22329
Sulfo-SIAB	Amine/Sulfhydryl	22327

Pyridyl Disulfides

Pyridyl disulfides react with sulfhydryl groups over a broad pH range (optimal pH is 4-5) to form disulfide bonds, and therefore, conjugates prepared using these reagents are cleavable. During the reaction, a disulfide exchange occurs between the molecule's –SH group and the 2-pyridyldithiol group. As a result, pyridine-2-thione is released and its concentration can be determined by measuring the absorbance at 343 nm (Figure 6). These reagents can be used as cross-linkers and to introduce sulfhydryl groups into proteins. The disulfide exchange can be performed at physiological pH, although the reaction rate is slower. (See Table 3 for the pyridyldithiol cross-linkers available from Pierce.)

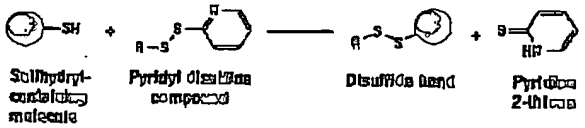


Figure 6. Pyridyl disulfide reaction scheme. Reaction efficiency can be monitored by determining the concentration of the released pyridine-2-thione by measuring the absorbance at 343 nm (molar extinction coefficient at 343 nm = $8.08 \times 10^3\ M^{-1}cm^{-1}$).

Table 3. Available Pyridyl Disulfide Cross-linkers

Reagent	Reactivity	Product #
LC-SPDP	Sulfhydryl/Amine	21551
Sulfo-LC-SPDP	Sulfhydryl/Amine	21650
PDPH	Sulfhydryl/Carbohydrate	22301

Hydrazides

Carbonyls (aldehydes and ketones) react with hydrazides and amines at pH 5-7. The reaction with hydrazides is faster than with amines, making them useful for site-specific cross-linking. Carbonyls do not readily exist in proteins; however, mild oxidation of sugar glycols using sodium *meta*-periodate will convert vicinal hydroxyls to aldehydes or ketones (Figure 7). The oxidation is performed in the dark at 0–4°C to prevent side reactions. Subsequent reaction with hydrazides results in formation of a hydrazone bond. Carbohydrate modification is particularly useful for antibodies in which the carbohydrate is located in the Fc region away from binding sites. At 1 mM $NaIO_4$ and a temperature of 0°C, the oxidation is restricted to sialic acid residues. At concentrations of 6-10 mM periodate, other carbohydrates in proteins (including antibodies) will be targeted.

Oxidation of a carbohydrate (cis-diol) to an aldehyde

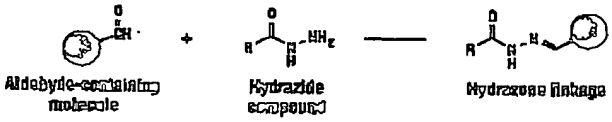


Figure 7. Hydrazide reaction schema.

Carbodiimides

Carbodiimides couple carboxyls to primary amines or hydrazides resulting in the formation of amide or hydrazone bonds. Carbodiimides are unlike other conjugation reactions in that no spacer exists between the molecules being coupled. Carboxy termini of proteins can be targeted, as well as glutamic and aspartic acid side-chains. In the presence of excess cross-linker, polymerization is likely to occur because all proteins contain carboxyls and amines. The bond that results is the same as a peptide bond, so reversal of the conjugation is impossible without destroying the protein.

EDC (Product # 22980, 22981) reacts with carboxylic acid group and activates the carboxyl group to form an active *O*-acylisourea intermediate, allowing it to be coupled to the amino group in the reaction mixture. An EDC by-product is released as a soluble urea derivative after displacement by the nucleophile (Figure 8). The *O*-acylisourea intermediate is unstable in aqueous solutions, making it ineffective in two-step conjugation procedures without increasing the stability of the intermediate using *N*-hydroxysuccinimide. This intermediate reacts with a primary amine to form an amide derivative. Failure to react with an amine results in hydrolysis of the intermediate, regeneration of the carboxyls, and the release of an N-unsubstituted urea. The cross-linking reaction is usually performed between pH 4.5 to 5 and requires only a few minutes for many applications. However, the yield of the reaction is similar at pH from 4.5 to 7.5.

The hydrolysis of EDC is a competing reaction during coupling and is dependent on temperature, pH and buffer composition. 4-Morpholinoethanesulfonic acid (MES, Product # 28390) is an effective carbodiimide reaction buffer. Phosphate buffers reduce the reaction efficiency of the EDC, but increasing the amount of EDC can compensate for the reduced efficiency. Tris, glycine and acetate buffers may not be used as conjugation buffers. NHS (Product # 24500) or its water-soluble analog Sulfo-NHS (Product # 24510), is often included in EDC coupling protocols to improve efficiency. EDC couples NHS to carboxyls, resulting in an NHS-activated site on a molecule. The NHS-ester formed and the carbodiimide's *O*-acylisourea intermediate are amine-reactive; however, an NHS-ester has much greater stability in slightly acidic or near neutral pH conditions. In water, an NHS-ester has a half-life of one to several hours, or even days, depending on temperature, pH and structure of the cross-linker, but *O*-acylisourea intermediate has a half-life measured in seconds in acidic or neutral pH conditions. EDC-mediated coupling of molecules works well in many applications, without the addition of NHS or Sulfo-NHS, which are not generally required unless protein concentrations are very low. When a large excess of EDC is used without NHS, it is often necessary to reduce the EDC amount when converting to an EDC/NHS system to prevent excessive cross-linking and possible precipitation.

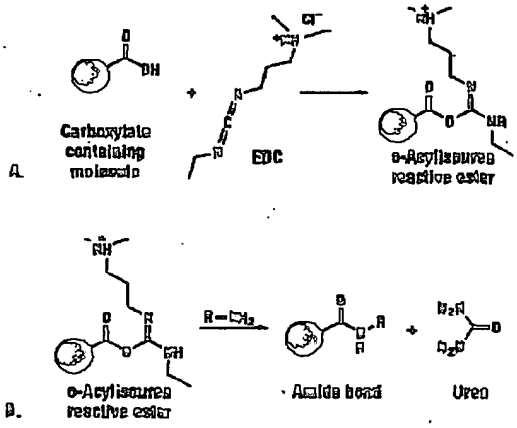


Figure 8. EDC coupling reaction schema.

Aryl Azides

Photoreactive reagents are chemically inert reagents that become reactive when exposed to ultraviolet or visible light. With few exceptions, the photoreactive groups in these reagents are aryl azides (Figure 9). When an aryl azide is exposed to UV light, it forms a nitrene group that can initiate addition reactions with double bonds, insertion into C-H and N-H sites, or subsequent ring expansion to react with a nucleophile (e.g., primary amines, Figure 10). The latter reaction path dominates when primary amines are present in the sample. Thiol-containing reducing agents (e.g., DTT or 2-mercaptoethanol) must be avoided in the sample solution during all steps before and during photoactivation. These reagents will reduce the azide functional group to an amine, preventing photoactivation. Reactions can be performed in a variety of amine-free buffer conditions. If working with heterobifunctional photoreactive cross-linkers, use buffers compatible with the chemically reactive portion of the reagent. The chemical reaction is performed in subdued light with reaction vessels covered in foil. The photoactivation can be performed with a bright camera flash or ultraviolet hand-held lamp about 1-3 inches above the reaction vessels. A bright camera flash works well with the nitro- and hydroxyl-substituted aryl azides. Unsubstituted aryl azides may require ultraviolet light or numerous flashes.

Protein Structure – Cross-linking

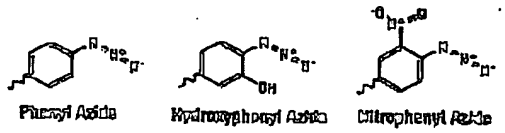


Figure 9. Forms of aryl azide reactive groups in photoreactive cross-linking reagents.

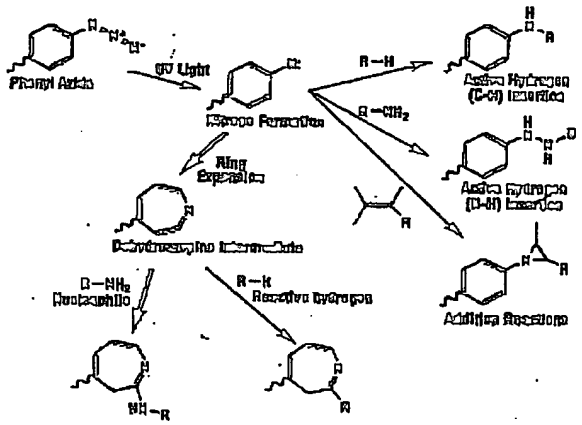


Figure 10. Possible reaction pathways of aryl azide cross-linkers.

Table 4. Available Aryl Azide Cross-linkers

		Reactive Group	
Reagent	Product #	Photoreactive	Other Group(s)
ABH	21510	Phenyl azide	Hydrazide
ANB-NOS	21451	Nitrophenyl azide	NHS
APDP	27720	Hydroxyphenyl azide	Pyridyldisulfide
APG	20108	Phenyl azide	Phenylglyoxal
ASBA	21512	Hydroxyphenyl azide	Amine
BASED	21564	Hydroxyphenyl azide	Hydroxyphenyl azide
HNS-ASA	27714	Hydroxyphenyl azide	NHS
SADP	21533	Phenyl azide	NHS
SAED	33030	Azido-methylcoumarin	Sulfo-NHS
SAND	21549	Nitrophenyl azide	Sulfo-NHS
SANPAH	22600	Nitrophenyl azide	NHS
SASD	27718	Hydroxyphenyl azide	Sulfo-NHS
SFAD	27719	Perfluorophenyl azide	Sulfo-NHS
SPB	23013	Psoralen	NHS
Sulfo-HSAB	21563	Phenyl azide	Sulfo-NHS
Sulfo-NHS-LC-ASA	27720	Hydroxyphenyl azide	Sulfo-NHS
Sulfo-SADP	21563	Phenyl azide	Sulfo-NHS
Sulfo-SANPAH	22589	Nitrophenyl azide	Sulfo-NHS
Sulfo-SBED	33033	Phenyl azide	Sulfo-NHS/Biotin

Cross-linking Applications

Cell Surface Cross-linking

Cross-linkers are often used to identify surface receptors or their ligands. Membrane-impermeable cross-linkers ensure cell-surface specific cross-linking. Water-insoluble cross-linkers when used at controlled amounts of reagent and reaction time can reduce membrane penetration and reaction with inner membrane proteins.

The sulfonyl group attached to the succinimidyl rings of a NHS-esters result in a cross-linker that is water-soluble, membrane-impermeable and non-reactive with inner membrane proteins. Therefore, reaction time and quantity of cross-linker are less critical when using sulfo-NHS-esters. Homobifunctional sulfo-NHS-esters, heterobifunctional sulfo-NHS-esters and photoreactive phenyl azides are good choices for cross-linking proteins on the cell surface.

Determination of whether a particular protein is located on the surface or the integral part of the membrane can be achieved by performing a conjugation reaction of a cell membrane preparation to a known protein or radioactive label using a water-soluble or water-insoluble cross-linker. Upon conjugation the cells may be washed, solubilized and characterized by SDS-polyacrylamide gel electrophoresis (PAGE) to determine whether the protein of interest was conjugated. Integral membrane proteins will form a conjugate in the presence of a water-insoluble cross-linker, but not in the presence of water-soluble cross-linkers. Surface membrane proteins can conjugate in the presence of water-soluble and water-insoluble cross-linkers. BASED (Product # 21564), a homobifunctional photoactivatable phenyl azide, is one of the more versatile cross-linkers for the study of protein interactions and associations. It is cleavable and can be radiolabeled with ^{125}I using IODO-BEADS® Iodination Reagent (Product # 28665). After cleavage, both of the dissociated molecules will still be iodinated. Because both reactive groups on this cross-linker are nonspecific, the cross-linking is not dependent on amino acid composition for successful conjugation.

Arginine-Specific Cross-linking

Glyoxals are useful compounds for targeting the guanidinyl portion of arginine residues. Glyoxals will target arginines at mildly alkaline pH. There is some cross-reactivity (the greatest at pH ≥9) with lysines. An example of this type of linker is APG (Product # 20108), which has an aryl azide moiety in addition to the glyoxal group. This cross-linker is most useful for targeting compounds deficient in primary amines.

Cell Membrane Structural Studies

Cell membrane structural studies require reagents of varying hydrophobicity to determine the location and the environment within a cell's lipid bilayer. Fluorescent tags are used to locate proteins, lipids or other molecules inside and outside the membrane. Various cross-linkers with differing spacer arm lengths can be used to cross-link proteins to associated molecules within the membrane to determine the distance between molecules. Successful cross-linking with shorter cross-linkers is a strong indication that two molecules are interacting in some manner. Failure to obtain cross-linking with a panel of shorter cross-linkers, while obtaining conjugation with the use of longer reagents, generally indicates that the molecules are located in the same part of the membrane but are not interacting. Homobifunctional NHS-esters, imidates or heterobifunctional NHS-ester/photoactivatable phenyl azides are commonly used for these procedures. Although imidoester cross-linkers (imidates) are water-soluble, they are still able to penetrate membranes. Sulfhydryl-reactive cross-linkers may be useful for targeting molecules with cysteines to other molecules within the membrane.

EDC (Product # 22980, 22981), water-insoluble dicyclohexylcarbodiimide (DCC, Product # 20320), and other water-soluble/-insoluble coupling reagent pairs are used to study membranes and cellular structure, protein subunit structure and arrangement, enzyme:substrate interactions, and cell surface and membrane receptors. The hydrophilic character of EDC can result in much different cross-linking patterns in

membrane and subunit studies than with hydrophobic carbodiimides such as DCC. Often it is best to attempt cross-linking with a water-soluble and water-insoluble carbodiimide to obtain a complete picture of the spatial arrangements or protein:protein interactions involved.

Subunit Cross-linking and Protein Structural Studies

Cross-linkers can be used to study the structure and composition of proteins in samples. Some proteins are difficult to study because they exist in different conformations with varying pH or salt conditions. One way to avoid conformational changes is to cross-link subunits. Amine-, carboxyl- or sulfhydryl-reactive reagents are used for identification of particular amino acids or for determination of the number, location and size of subunits. Short-to-medium spacer arm cross-linkers are selected when intramolecular cross-linking is desired. If the spacer arm is too long, intermolecular cross-linking can occur. Carbodiimides that result in no spacer arm, along with short length conjugating reagents, such as amine-reactive DFDNB (Product # 21525) or the photoactivatable amine-reactive cross-linker NHS-ASA (Product # 27714), can cross-link between subunits without cross-linking to extraneous molecules if used in optimal concentrations and conditions. Slightly longer cross-linkers, such as DMP (Product # 21666, 21667) can also cross-link between subunits, but they may result in intermolecular coupling. Adjusting the reagent amount and protein concentration can control intermolecular cross-linking. Dilute protein solutions and high concentrations of cross-linker favor intramolecular cross-linking when homobifunctional cross-linkers are used.

Non-cleavable, homobifunctional, sulfhydryl-reactive linkers such as BMH (Product # 22330) can be used to link subunits that were joined by disulfide bonds. In some circumstances, the cross-linking pattern or success may be affected by the cross-linker's solubility. Hydrophobic cross-linkers tend to cross-link more effectively in hydrophobic regions of molecules.

For determination or confirmation of the three-dimensional structure, cleavable cross-linkers with increasing spacer arm lengths may be used to determine the distance between subunits. Experiments using cross-linkers with different reactive groups may indicate the locations of specific amino acids. Once conjugated, the proteins are subjected to two-dimensional electrophoresis. In the first dimension, the proteins are separated using non-reducing conditions and the molecular weights are recorded. Some subunits may not be cross-linked and will separate according to their individual molecular weights. Conjugated subunits will separate according to the combined molecular weight. The second dimension of the gel is then performed using conditions to cleave the cross-linked subunits. The individual molecular weights of the cross-linked subunits can be determined. Cross-linked subunits that were not reduced will produce a diagonal pattern, but the cleaved subunits will be off the diagonal. The molecular weights of the individual subunits should be compared with predetermined molecular weights of the protein subunits using reducing SDS-polyacrylamide gel electrophoresis.

Intermolecular Cross-linking for the Study of Protein Interactions and Associations

Cross-linkers are used for identification of near-neighbor protein relationships and ligand-receptor identification and interactions. The cross-linkers chosen for these applications are usually longer than those used for subunit cross-linking. Homobifunctional, amine-reactive NHS-esters or imidates and heterobifunctional, amine-reactive, photoactivatable phenyl azides are the most commonly used cross-linkers for these applications. Occasionally, a sulfhydryl- and amine-reactive cross-linker, such as Sulfo-SMCC (Product # 22322) may be used if one of the two proteins or molecules is known to contain sulfhydryls. Both cleavable or noncleavable cross-linkers can be used. Because the distances between two molecules are not always known, the optimal length of the spacer arm of the cross-linker may be determined by the use of a panel of similar cross-linkers with different lengths. DSS (Product # 21555) or its cleavable analog DSP (Product # 22585) are among the shorter cross-linkers used for protein:protein interactions. NHS-ester, phenyl azides are very useful for this type of cross-linking because they usually result in efficient cross-linking. SASD (Product # 27716) is a unique sulfo-NHS-ester, photoactivatable phenyl azide that is both iodinatable and cleavable that allows for detection and analysis of small quantities of protein. For more information on this type of application for cross-linkers, refer to catalog Section 11: Protein Interactions.

Cross-linkers for Immunotoxin Production

Specific antibodies can be covalently linked to toxic molecules and then used to target antigens on cells. Often these antibodies are specific for tumor associated antigens. Immunotoxins are brought into the cell by surface antigens and, once internalized, they proceed to kill the cell by ribosome inactivation or other means. The type of cross-linker used to make an immunotoxin can affect its ability to locate and kill the appropriate cells. For immunotoxins to be effective, the conjugate must be stable *in vivo*. In addition, once the immunotoxin reaches its target, the antibody must be separable from the toxin to allow the toxin to kill the cell. Thiol-cleavable, disulfide-containing conjugates have been shown to be more cytotoxic to tumor cells than noncleavable conjugates of ricin A immunotoxins. Cells are able to break the disulfide bond in the cross-linker, allowing the release of the toxin within the targeted cell.

SPDP (Product # 21857) is a reversible NHS-ester, pyridyl disulfide cross-linker used to conjugate amine-containing molecules to sulfhydryls. For several years, this has been the "workhorse" cross-linker for production of immunotoxins. The amine-reactive NHS-ester is usually reacted with the antibody first. In general, toxins do not contain surface sulfhydryls; therefore, sulfhydryls must be introduced onto them by reduction of disulfides, which is common for procedures involving ricin A chain and abrin A chain, or through chemical modification reagents. A second SPDP molecule can be used for this purpose and is reacted with amines on the immunotoxin, then reduced to yield sulfhydryls. Another chemical modification reagent that is commonly used for production of immunotoxins is 2-iminothiolane, also known as Traut's Reagent (Product # 26101). Traut's Reagent reacts with amines and yields a sulfhydryl when its ring structure opens during the reaction.